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ORIGINAL ARTICLE

Massive cellular disruption occurs during early imbibition of *Cuphea* seeds containing crystallized triacylglycerols

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Abstract The transition from anhydrobiotic to hydrated state occurs during early imbibition of seeds and is lethal if lipid reserves in seeds are crystalline. Low temperatures crystallize lipids during seed storage. We examine the nature of cellular damage observed in seeds of *Cuphea wrightii* and *C. lanceolata* that differ in triacylglycerol composition and phase behavior. Intracellular structure, observed using transmission electron microscopy, is profoundly and irreversibly perturbed if seeds with crystalline triacylglycerols are imbibed briefly. A brief heat treatment that melts triacylglycerols before imbibition prevents the loss of cell integrity; however, residual effects of cold treatments in *C. wrightii* cells are reflected by the apparent coalescence of protein and oil bodies. The timing and temperature dependence of cellular changes suggest that damage arises via a physical mechanism, perhaps as a result of shifts in hydrophobic and hydrophilic interactions when triacylglycerols undergo phase changes. Stabilizers of oil body structure such as oleosins that rely on a balance of physical forces may become ineffective when triacylglycerols crystallize. Recent observations linking poor oil body stability and poor seed storage behavior are potentially explained by the phase behavior of the storage lipids. These findings directly impact the feasibility of preserving genetic resources from some tropical and subtropical species.

Keywords *Cuphea* · Differential scanning calorimetry · Imbibitional damage · Intermediate seeds · Liposome · Recalcitrant seeds

Abbreviations
DSC Differential scanning calorimetry
GRIN Germplasm resources information network
TEM Transmission electron microscopy

Introduction

Water interactions with crystallized storage lipids are lethal to seeds (Crane et al. 2003, 2006) and may cause a syndrome known as intermediate storage behavior (Ellis et al. 1990). Seeds exhibiting this storage behavior survive drying to very low water contents, but do not survive low temperature storage or the combined effects of drying and cooling. Seeds in this category usually originate from tropical or subtropical regions and often have storage lipids that crystallize above −10°C and melt at temperatures above 25°C, respectively. A simple heat exposure that melts the triacylglycerols before seeds imbibe eliminates evidence of damage, and allows seeds to germinate normally (Crane et al. 2003, 2006).

This publication is dedicated in memory of Dr. Vincent Franceschi, a mentor and friend. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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The reason why water contacting crystallized triacylglycerols is lethal to seeds is not known. Crystallization and melting of storage lipids are completely reversible in dry seeds, as is evident from the routine storage of many crop species (so-called orthodox seeds) at liquid nitrogen temperatures (< −135°C; Stanwood and Bass 1981; Walters et al. 2004). We have used principles developed from the imbibitional chilling injury literature (Crowe et al. 1989; Hoekstra et al. 2001; Sacandé et al. 2001; Golovina and Hoekstra 2003) and from the freeze-desiccation damage literature (Crowe and Crowe 1992; Steponkus et al. 1995) to hypothesize about the mechanisms of damage induced when seeds with crystallized triacylglycerols are imbibed (Crane et al. 2003, 2006). These hypotheses may be explained by decompartmentalization within cells because of altered hydrophilic and hydrophobic interactions on the liposome membrane.

Our understanding of membrane structure of liposomes primarily arises from elegant studies describing the function of oleosins within seeds. Oleosins are structural proteins with a molecular mass of 15–26 kDa that are believed to stabilize lipid bodies within cells by integrating within the surrounding lipid monolayer (Fernandez et al. 1988; Ross and Murphy 1992; Wang et al. 1997; Hsieh and Huang 2004). During seed maturation, storage lipids are deposited into the endoplasmic reticulum and oleosins help constrain vesicle size during lipid body biogenesis (Ting et al. 1996). During germination, storage lipids are mobilized and the constrained size of the lipid body is believed to increase efficiency of catalysis by lipase (Fernandez and Staehelin 1987; Huang 1992; Herman 1995; Murphy et al. 2001). An additional role of stabilizing liposome structure during imbibition has been attributed to oleosins based on studies showing lipid body coalescence in seeds that do not store well, such as cacao and neem (Leprince et al. 1998; Murphy et al. 2001; Neya et al. 2004). This hypothesis was recently challenged when oleosins were detected in cacao seeds (Guilloteau et al. 2003). Seeds having recalcitrant or intermediate storage physiologies often contain lipids that melt at relatively high temperatures, an observation that provides an intriguing link between lipid composition, liposome stability and storage physiology.

In this paper, we investigate the cellular damage incurred during imbibition of seeds containing crystallized lipids. We use seeds from the species *Cuphea* (Lythraceae) as a model system because the range of fatty acid compositions produced among seeds within this genus (Wolf et al. 1983; Widrlechner and Kovach 2000) allows us to study the effects of interactions between water and crystallized lipid at physiological temperatures (Crane et al. 2003, 2006). Lipids extracted from *Cuphea* are useful in the production of soaps, detergents, surfactants, lubricants and confections, and development of *Cuphea* as a crop may reduce US dependence on oils from tropical plants (Thompson 1984; Graham 1989). Here, we compare two species, *C. wrightii* and *C. lanceolata*, which produce seeds with different proportions of capric (10 carbon chain, C10) and lauric (12 carbon chain, C12) acids. *C. wrightii* is an early successional species found in disturbed, wet habitats of southwest and as far south as Costa Rica (Graham 1988, 1989). Seeds of *C. wrightii* contain high amounts of lauric acid and are killed following low temperature storage and imbibition at room temperature (Crane et al. 2003). *C. lanceolata* is native to northern and central Mexico and seeds of this species, which contain high amounts of capric acid, germinate normally after low temperature storage and imbibition at room temperature, but are killed if imbibed at temperatures less than 15°C (Crane et al. 2003).

Materials and methods

Plant materials and lipid properties

Seeds of *C. wrightii* (PI 594955) and *C. lanceolata* (PI 594931) were grown and harvested at the USDA-ARS North Central Regional Plant Introduction Station (NCRPIS) in Ames (IA, USA) in 2001. Seeds were stored at 4°C until 2003, when they were sent to the USDA-ARS National Center for Genetic Resources Preservation in Fort Collins (CO, USA). In Fort Collins, seeds were stored at 22°C until their use in 2005. Prior to use, seeds were given a 1 h exposure to 45°C to ensure that all lipids were fully melted at the onset of all experiments (Crane et al. 2003).

Fatty acid composition was determined for the total and polar lipid fractions within seeds of both species. Lipids were extracted from powdered seeds using a 2:1 solution of chloroform: methanol (Bligh and Dyer 1959). The lipid fraction was dissolved in chloroform and polar lipids were separated from the total lipid fraction using solid-phase extraction cartridges (Sep-pak Silica; Waters, Milford, MA, USA). Fatty acid methyl esters were prepared using 12% boron-trifluoride (Metcalfe and Schmitz 1961) and were characterized using a Perkin-Elmer 8500 gas chromatograph equipped with an FID detector and a Supelco Nukol 30 m, 0.25 mm internal diameter fused silica capillary column using previously described methods (Crane et al. 2003).

Crystallization and melting transitions within *C. wrightii* and *C. lanceolata* seeds were measured using differential scanning calorimetry (DSC; Perkin-Elmer DSC7). Four-milligram samples of whole seed were hermetically sealed into Perkin-Elmer volatile sample pans and loaded into the DSC, previously calibrated for temperature with methylene chloride (−95°C) and indium (156.6°C). Heat flow was recorded as samples were cooled from 20 to −100°C and rewarmed from −100 to + 50°C using a scanning rate of 10°C min^{-1} throughout. Onset temperatures of the transitions were calculated using Perkin-Elmer software from the intersection of the baseline and the tangent to the steepest part of the transition peak.

Temperature and hydration treatments

The experimental design consisted of exposing dry seeds [0.05 g water (g dry mass)$^{-1}$] to low temperatures to induce crystallization in the triacylglycerol fraction, and then warming to various temperatures to control lipid melting before imbibition. Seeds of *C. lanceolata* were initially cooled to −80°C while seeds of *C. wrightii* were cooled to either −18 or −80°C in plastic Petri dishes placed into the appropriate freezer overnight. Following this low temperature treatment, the dry seeds were warmed to 5°C (in a refrigerator), 22°C (on the laboratory bench) or 45°C (in an incubator) for at least 1 h before imbibition treatments were started.

Seeds were imbibed on damp blotter paper at either 5 or 22°C. Samples that were initially imbibed at 5°C were transferred directly to 22°C after 4 or 24 h.

Viability tests

Following temperature and imbibition treatments described above, seeds were placed on damp blotter paper in Petri dishes at 25°C with a 16/8 light/dark cycle and allowed to germinate for up to six weeks. A seed was scored as germinated when both the radicle and hypocotyl emerged.

Tetrazolium tests (Peters 2000) were used to confirm germination results for some treatments. Seeds were imbibed on moist blotter paper at 22°C overnight, cut, and treated with 1% solution of buffered 2,3,5-triphenyl-tetrazolium chloride. Color development was observed in embryos after 8 h.

Viability was also assessed in embryonic axes that were separated from cotyledons. Following some temperature treatments, excised axes were sterilized for 10 min with a 10% bleach solution, rinsed with sterile water, and cultured on Linsmaier and Skoog basal medium (Linsmaier and Skoog 1965) with 30 g sucrose and 7 g agar at pH 5.9.

Conductivity measurements

Electrolyte leakage was determined using an automatic seed conductivity meter (ASAC 1000, Neogen Corporation, MI, USA). Five replicates of 15 mg of seed received the various temperature treatments and then were submerged in 2.0 ml of distilled and deionized water at 22°C. Conductivity measurements were taken every 5 min for an hour and leakage rate was determined for the period of most rapid increase in conductivity for each replicate (usually between 5 and 35 min). The results for the average rate are expressed as μA mg^{-1} h^{-1}.

Microscopy

Cellular structure was examined in seeds that had been given various combinations of the low temperature (−18 or −80°C for 24 h) and warming (5, 22 or 45°C for 1 h) treatments and then imbibed on moist blotter paper for 4 or 27 h at 5 or 22°C. After the imbibition period, seed coats were removed and embryos were cut into several pieces. In some experiments, embryonic axes were separated from cotyledons. Samples were fixed overnight in 1.25% glutaraldehyde and 2% paraformaldehyde in 0.05 M pipes buffer at 22°C. Tissue samples were rinsed three times in 0.05 M Pipes buffer and post-fixed with 2% osmium tetroxide in 0.05 M Pipes buffer for 24 h, under vacuum, at 22°C. After three rinses in Pipes buffer, samples were gradually dehydrated in a gradient acetone series from 30 to 100%. Samples were infiltrated with Spurr's resin (Electron Microscopy Sciences, Hatfield, PA, USA) and hardened at 95°C overnight. Thick sections were made with glass knives on a RMC MT-X ultramicrotome (Ventana Medical Systems Inc., Tucson, AZ, USA) and were stained with Stevenel's stain (del Cerro et al. 1980). Thin sections (70–90 μm) were cut with a diamond knife, mounted on formvar-coated copper grids (150 mesh) and stained with 3.2 % uranyl acetate in 50% methanol and 50% ethanol for 15 min and Reynold's lead citrate (Bozolla and Russell 1991) for 15 min. Thin sections were observed using a JEOL 2000 EXII transmission electron microscope (TEM; Jeol. Ltd. Tokyo, Japan) at 100 kV accelerating voltage. Evidence of cellular damage was assessed on thick sections (1 μm) using light microscopy and confirmed in TEM observations of thin sections. The fraction of disrupted cells was calculated from observations of three regions within a minimum of three samples for each species and temperature-imbibition combination. Each experiment was repeated once and data within replicate experiments were combined. Protein storage

vacuole and lipid body cross-sectional areas were digitally quantified from micrographs using Image Pro+ software (Media Cybernetics, Inc., Silver Spring, MD, USA). Cross-sectional areas of organelles were statistically compared using the JMP software package (SAS Institute Inc., Cary, NC, USA).

Results

Lipid properties

This paper compares the cellular disruption that occurs during imbibition of seeds from two species of *Cuphea* that differ in the fatty acid composition of their storage and polar lipids. Lipid compositions of the accessions used in this study are similar to values reported for the species on the Genetic Resources Information Network (GRIN; Table 1). Seeds of *C. wrightii* and *C. lanceolata* contain 38 ± 1% and 33 ± 1% lipid (dry mass basis), respectively. Greater than 60% of the fatty acids in *C. wrightii* are lauric (C12) or myristic (C14) acids, and 28% are capric acid (C10). In contrast, only 5% of the fatty acids in seeds of *C. lanceolata* are lauric or myristic acids while 75% are capric acid (Table 1). The polar lipid fraction within both species contained higher proportions of longer chain and unsaturated fatty acids compared to the total lipid fraction.

The DSC scans of *C. wrightii* seeds have higher crystallization and melting temperatures compared to those of *C. lanceolata* seeds, which is consistent with the relative proportions of lauric and myristic acids. According to DSC thermograms of seeds, crystallizations within *C. wrightii* and *C. lanceolata* occurred at −8 and −28°C, respectively (Fig. 1a). Melting transitions began at 22 and 13°C, respectively, and the temperatures at the peak of the melting transitions were 31 and 20°C, respectively (Fig. 1b). The melting transition temperatures measured using DSC were similar to calculations based on weighted averages of (β′ crystal melting temperatures of simple triacylglycerols (Table 1). Gel to liquid crystalline phase temperatures of the polar lipid fractions were predicted at 0 and −1°C for *C. wrightii* and *C. lanceolata*, respectively, based on weighted averages of hydrated diacylcerolphosphotidylcholines with the indicated fatty acid compositions (Table 1). These composition and thermal data indicate that lipids in *Cuphea* seeds crystallize when seeds are cooled to −80°C, and that membranes within both species will likely become fluid during hydration at 0°C or above, but that triacylglycerols will remain crystallized at temperatures below about 30°C for *C. wrightii* or 20°C for *C. lanceolata* seeds. Thus,

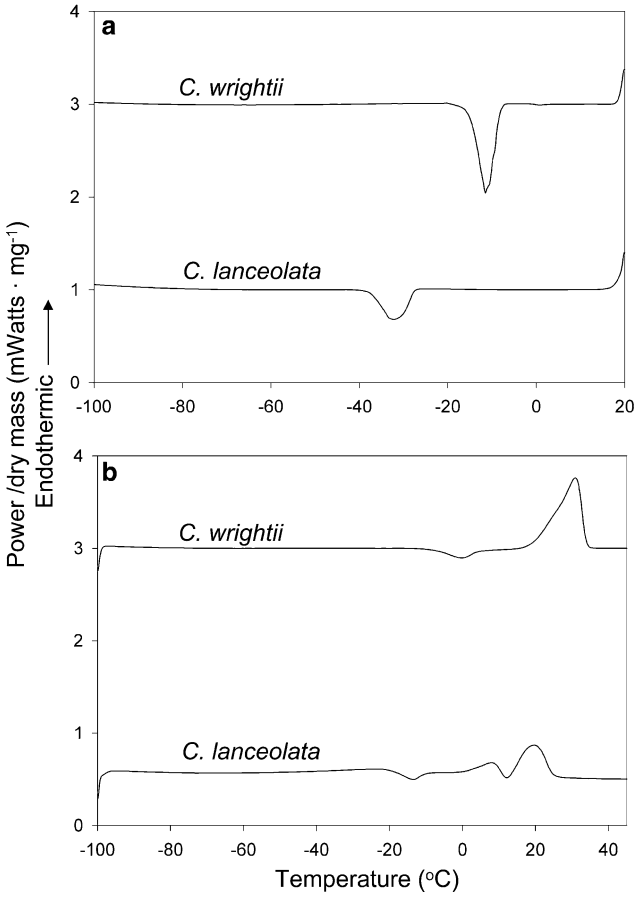

Fig. 1 Cooling (**a**) and warming (**b**) thermograms of *Cuphea wrightii* and *C. lanceolata* seeds measured using differential scanning calorimetry. Crystallization transitions are the exothermic events that occur upon cooling and melting transitions are the endothermic events that occur upon warming. Transition temperatures and enthalpies are consistent with triacylglycerol composition within the seeds

after seeds were cooled to −80°C, our experiments targeted warming and imbibition temperatures of 5°C at which membranes are supposedly fluid but triacylglycerols remain crystallized for both species, 22°C at which triacylglycerols are fluid in *C. lanceolata* but not in *C. wrightii*, and 45°C at which triacylglycerols are fluid in both species.

Imbibition at 22°C

Viability of seeds corresponded to postulated triacylglycerol phases during initial imbibition. Seeds from both species exhibited high germination, successful axis culture, and positive tetrazolium staining if they were not previously exposed to −80°C (Table 2). Germination and axis culture (at 25°C) of *C. wrightii* seeds was near 0% after exposure to −80°C while most seeds of *C. lanceolata* germinated normally if warmed

Table 1 Fatty acid composition of total and polar lipid fractions within seeds of *Cuphea wrightii* and *C. lanceolata* and its effect on melting temperature

Fatty acid	Chain length	Temperature of melt (°C)[b]		Lipid composition (%)					
				C. wrightii			*C. lanceolata*		
		Triacyl (B')	Polar (gel)	GRIN[a]	Total	Polar	GRIN[a]	Total	Polar
Caprylic	C8	−21		1	0	0	1	0	0
Capric	C10	18	-15	28	14	14	75	75	0
Lauric	C12	35	0	56	68	53	2	3	3
Myristic	C14	47	23	5	11	3	3	3	3
Palmitic	C16	57	42	2	3	14	4	5	30
Stearic	C18	64	54	0	0	0	1	2	4
Oleic	C18:1	−12	−20	0	1	5	0	5	18
Linoleic	C18:2	−23	−30	6	3	11	7	7	42
Linolenic	C18:3	−34	−35	0	0	0	0	0	0
Sum				98	100	100	93	100	100
Predicted melt temperature (°C)[b]					32.3	0		17.7	−0.8

[a] Value obtained online by searching for data available for *Cuphea wrightii* and *Cuphea lanceolata* accessions on the Genetic Resources Information Network (http://www.ars-grin.gov/npgs/)

[b] Predicted temperature of lipid melt based on weighted average of transition temperatures for simple triacylglycerols and hydrated diacylcerol-phosphotidylcholines with given fatty acid side chains. The data for simple triacylglycerols and diacylcerol-phosphotidylcholines in excess water are taken from Small (1986)

to 22°C or above before imbibition (Table 2). Dry seeds of *C. wrightii* initially exposed to subzero temperatures must be pretreated at 45°C before imbibition to restore germination (Table 2).

The data in Table 2 demonstrate that temperature treatments to control triacylglycerol phase prior to seed imbibition resulted in predictable changes to seed viability. Light microscopy and TEM were used to examine the nature of cellular perturbations and whether their incidence corresponded to patterns of lost viability. The ultrastructure of cells in cotyledon and axes of *Cuphea* seeds that were not cooled to −80°C resembled that of other orthodox seeds; the massive accumulation of storage reserves obscured detection of other organelles in the cytoplasm (Ross and Murphy 1992; Farrant et al. 1997; Walters et al. 2005; Figs. 2a, b, 3a). Lipid bodies could be identified by the intact monolayer of polar lipid and protein completely encompassing a spherical, non-staining oil droplet. Triacylglycerols in micrographs are electron lucent, partly because osmium bonds poorly to saturated alkanes and partly because ethanol and acetone used during embedding extracted lipids (Mollenhauer 1993). There were also several large electron-dense spherical masses identified as protein storage vacuoles.

Consistent with viability assessments, remarkable cellular perturbations were observed in *C. wrightii* seeds after they were cooled to −80°C and then warmed to 22°C and imbibed for 4–27 h (Figs. 2c, d, 4a). Similar damage was also observed in *C. wrightii* seeds initially cooled to –18°C (Fig. 4, Table 3), but not in *C. lanceolata* seeds (data not shown), presumably because the lipids in *C. lanceolata* crystallize at temperatures < −18°C (Fig. 1). In damaged cells, evidence of liposome membranes and spherical protein storage vacuoles was lost, and the cytoplasm was mostly electron lucent, indicating loss of triacylglycerol compartmentation. Electron lucent protrusions appear to cut into irregularly shaped, dark staining masses that were likely remnants of protein storage vacuoles. In *C. wrightii*, 100% of the cells in seeds cooled to −80°C and then imbibed at 22°C exhibited visual signs of damage in light and electron micrographs (Table 2). The same type of cellular damage occurred in similar proportions of cells within both axes and cotyledons when seeds of this species were cooled to −18°C and imbibed at 22°C for 17 h (Table 3). Despite the intracellular damage apparent in electron micrographs of cold-treated *C. wrightii* seeds, cell shape remained intact. (Fig. 4a) and electrolyte leakage was the same as seeds that did not receive a low temperature treatment (Table 2). These observations suggest that the plasma membrane was intact in *C. wrightii* seeds cooled to −80°C and then warmed to 22°C before imbibition, since high levels of electrolyte leakage are expected when the plasma membrane ruptures (Posmyk et al. 2001; Modi 2005). Cellular perturbations were not observed in *C. lanceolata* seeds subjected to the same series of temperature treatments (Fig. 3b, Table 2). Conductivity measurements revealed

Table 2 Assessments of viability and cellular integrity of *Cuphea* seeds imbibed at 22 or 5°C after receiving a series of temperature treatments to control lipid phase

Species	Exposure temperatures for dry seeds (°C)		Imbibition condition		Lipid phase during imbibition	Viability assessment				
	Cooled to	Warmed to	Temp. (°C)	Duration (h)		Germination (%)	Axis culture (%)	Tetrazolium staining (%)	Electrolyte leakage ($\mu A\ mg^{-1}h^{-1}$)	Visual damage [a](%)
Cuphea wrightii	None	None	22	4	Fluid	74 ± 1	86	100	21 ± 5	10 ± 10
	−80	22	22	4	Crystal	0	9 ± 2	25	19 ± 7	100 ± 0
	−80	45	22	4	Fluid	73 ± 4	90 ± 3	100	23 ± 4	0
	None	None	5	4	Fluid	73 ± 3	Na[b]	Na	Na	0
	−80	5	5	4	Crystal	0	Na	Na	Na	25 ± 13
	−80	5	5	24	Crystal	0	Na	Na	Na	100 ± 0
	−80	5	5/22	4/24[d]	Crystal	0	Na	Na	29 ± 6	85 ± 10
	−80	45	5	4	Fluid	56 ± 6	Na	Na	Na	Na
Cuphea lanceolata	None	None	22	4	Fluid	81 ± 2	70	100	6 ± 1	0
	−80	22	22	4	Fluid	97 ± 2	89	100	6 ± 1	0
	−80	45	22	4	Fluid	96 ± 3	72	96	7 ± 3	0
	None	None	5	4	Fluid	80 ± 3	Na	Na	Na	0
	−80	5	5	4	Crystal	12 ± 12	Na	Na	Na	0
	−80	5	5	24	Crystal	0	Na	Na	Na	0
	−80	5	5/22	4/24[d]	Crystal	0	Na	Na	8 ± 2	100 ± 0[c]
	−80	45	5	4	Fluid	95 ± 1	Na	Na	Na	na

After the imbibition period, seeds were transferred to room temperature for viability measurements

[a] Percentage of cells exhibiting damage observed using light microscopy

[b] Not available

[c] Damage observed using transmission electron microscopy resembled that of a dying cell

[d] Seeds were imbibed for 4 h at 5°C seeds and then transferred to 22°C for leakage measurements (immediately) and embedding (after 24 h)

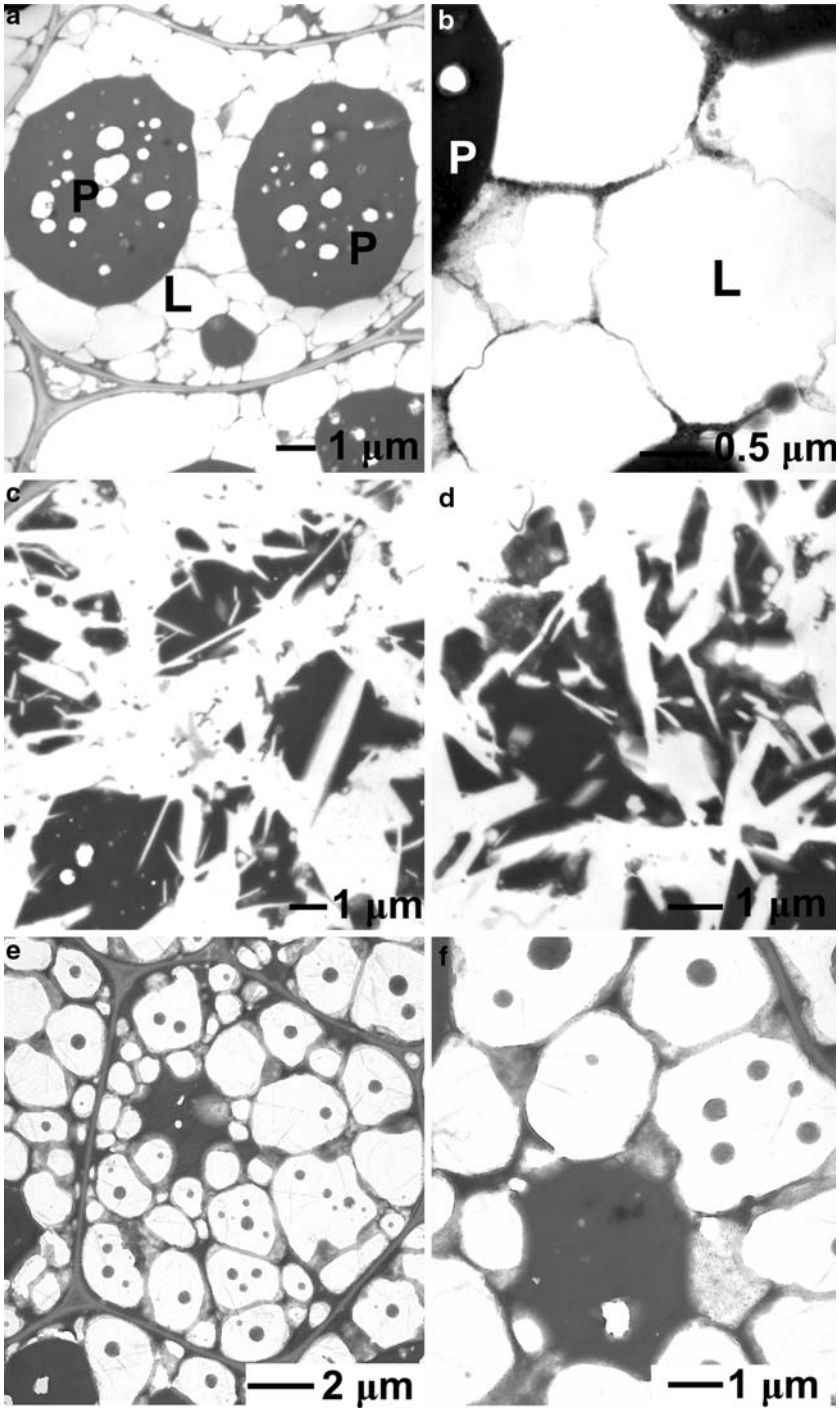

Fig. 2 Ultrastructure of cotyledon cells from *Cuphea wrightii* seeds after 4 h of imbibition at 22°C. **a**, **b** Seeds were not exposed to subzero temperatures before imbibition. **c**, **d** Seeds were cooled to −80°C overnight before imbibition at 22°C. **e**, **f** Seeds received the −80°C treatment and then a 1 h exposure to 45°C before imbibition at 22°C. *P* protein storage vacuole; *L* lipid body

three-fold lower electrolyte levels in leachate of *C. lanceolata* seeds compared to *C. wrightii* seeds, regardless of seed treatment (Table 2).

Warming seeds to 45°C before imbibition at 22°C restored high germination percentages in *C. wrightii* seeds that were cooled to −18 or −80°C (Table 2). Prewarmed seeds had organelles resembling protein and lipid bodies (Figs. 2e, f, 4b); however, the appearance of the storage reserves was substantially changed. The average cross-sectional area of the lipid bodies nearly doubled while the cross-sectional area of the protein storage vacuoles was halved in cells of *C. wrightii* seeds that were restored by the 45°C pulse (Fig. 5a, b). In contrast, the lipid body size in cells of *C. lanceolata* remained constant throughout temperature fluctuations, but the size of the protein storage vacuoles was reduced.

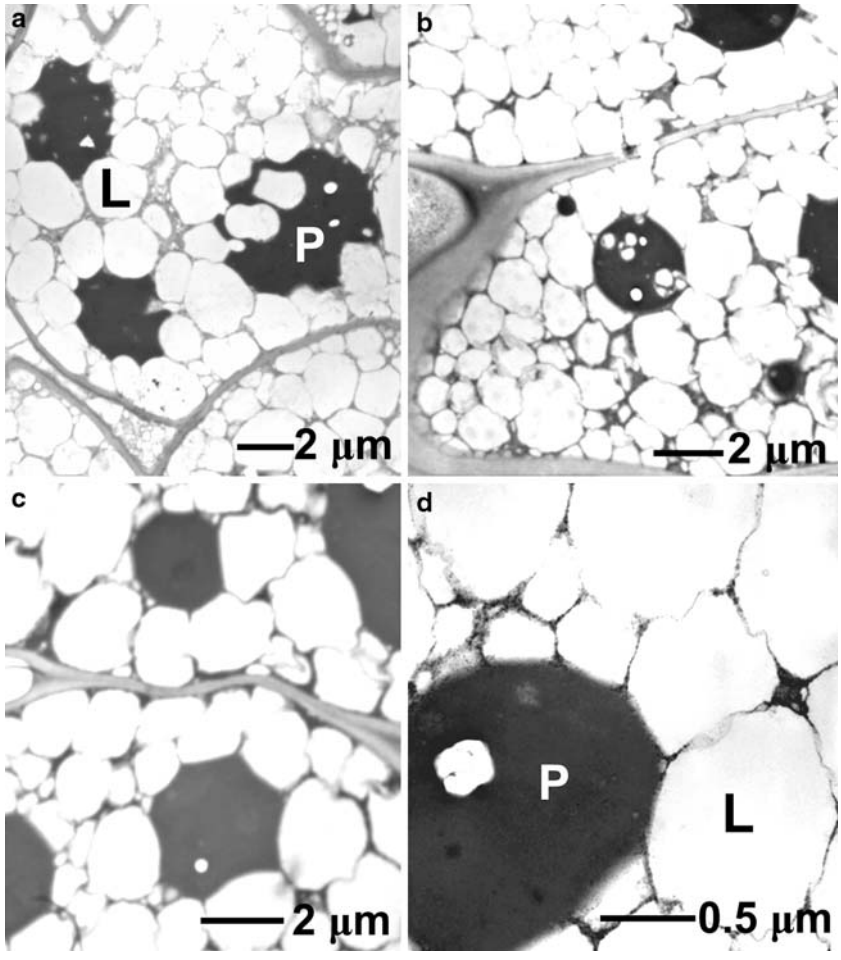

Fig. 3 Ultrastructure of cotyledon cells from *Cuphea lanceolata* after 4 h of imbibition at 22°C. **a** Seeds were not exposed to subzero temperatures before imbibition. **b** Seeds were cooled to −80°C overnight before imbibition at 22°C. **c**, **d** Seeds received the −80°C treatment and then a 1 h exposure to 45°C before imbibition at 22°C. *P* protein storage vacuole; *L* lipid body

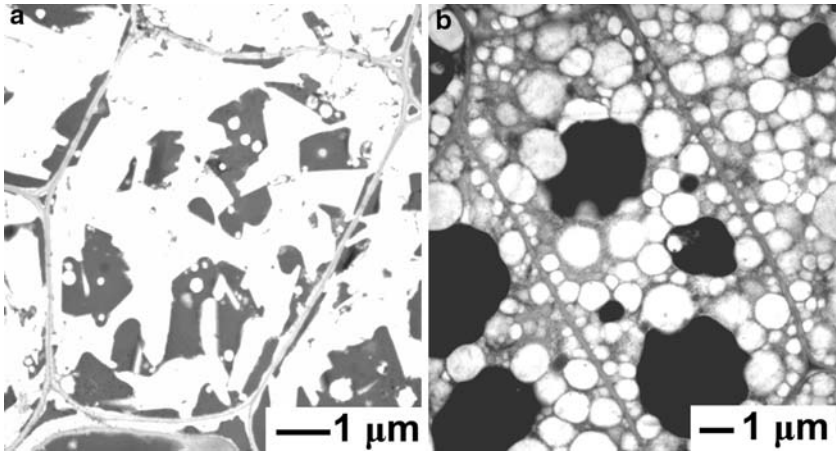

Fig. 4 Ultrastructure of cells of *Cuphea wrightii* seeds that were exposed to −18°C overnight and then imbibed 4 h at 22°C (**a**) or received a 1 h exposure to 45°C after the −18°C treatment and before the imbibition treatment (**b**)

Imbibition at 5°C

Imbibition of seeds at 5°C was not damaging per se for either species, as was demonstrated by normal germination percentages of seeds that did not receive a −80°C treatment and of seeds that received both a −80 and 45°C treatment prior to imbibition at 5°C (Table 2). However, when seeds were cooled to −80°C prior to imbibition at 5°C, viability decreased to 0 and 12% in seeds of *C. wrightii* and *C. lanceolata*, respectively (Table 2). Even a brief exposure of 4 h at 5°C during imbibition was sufficient to irreversibly reduce seed germination in both species (Table 2).

Visual evidence of cellular disruption in seeds exposed to −80°C and imbibed at 5°C was hardly apparent within 4 h of imbibition of either *C. wrightii*

Table 3 Cellular integrity within *Cuphea wrightii* seeds imbibed at 22°C after being cooled to −18°C to crystallize triacylglycerols and then warmed to 22 or 45°C to retain or melt, respectively, the lipid crystals

Exposure temperatures for dry seeds (°C)		Imbibition condition		Lipid phase during imbibition	Visual damage (%)[a]	
Cooled to	Warmed to	Temp. (°C)	Duration (h)		Axes	Cotyledons
none	none	22	17	crystal	0	0
−18	22	22	17	fluid	70 ± 30	90 ± 10
−18	45	22	17	crystal	0	3 ± 1

Cells were embedded after 4 h imbibition

[a] Percentage of cells exhibiting damage observed using light microscopy

or *C. lanceolata* (Figs. 6 a, b, 5 a, b, Table 2), but was obvious within 24 h of imbibition at 5°C (Fig. 6 c, d, Table 2). Cells of *C. wrightii* imbibed for 24 h at a constant 5°C lacked lipid and protein storage vacuoles with regular, spherical structure (Fig. 6c). Massive cell damage with similar characteristics to those depicted in Figs. 2c, 2d, and 4a was observed in seeds of *C. wrightii* that were imbibed for 4 h at 5°C and then transferred to 22°C for an additional 24 h of imbibition (Fig. 6e, cf 5). Evidence of lipid body fusion and disruption of cellular structures were also observed in cells of *C. lanceolata* seeds that were imbibed for 24 h (Fig. 6d) or 7 days (data not shown) at a constant 5°C. The loss of organelles and the presence of granulated masses were evidence that these cells were dying; however, cells of *C. lanceolata* seeds did not exhibit the striking cellular perturbations observed within *C. wrightii* seeds. Despite low viability (Table 2), cell structure seemed to be maintained in *C. lanceolata* seeds that were briefly imbibed at 5°C and then transferred to 22°C for an additional 24 h (Figs. 5, 6f, Table 2). The slow development of visual damage in seeds imbibed at 5°C suggests that the observed cellular disruptions are both time and temperature dependent.

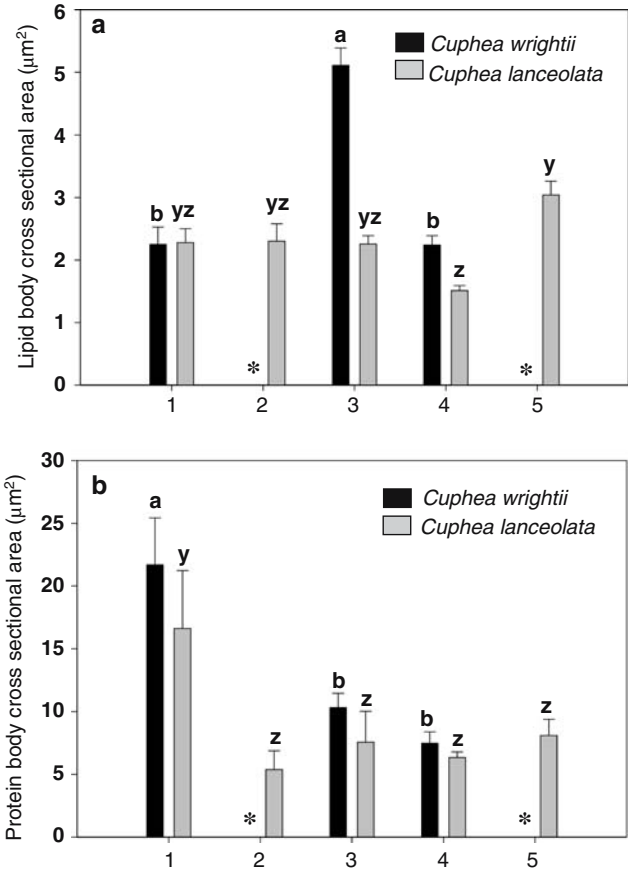

Fig. 5 Average cross sectional areas (± SE) of lipid bodies (**a**) and protein storage vacuoles (**b**) measured in *Cuphea wrightii* and *C. lanceolata* cells from electron micrographs similar to those presented in Figs. 2, 3, 4, 6. *Column 1* seeds were not cooled prior to 4 h imbibition at 22°C; *column 2* seeds were cooled to −80°C prior to 4 h imbibition at 22°C; *column 3* seeds were cooled to −80°C and then warmed to 45°C prior to 4 h imbibition at 22°C; *column 4* seeds were cooled to −80°C and then imbibed for 4 h at 5°C; *column 5* seeds were cooled to −80°C and then imbibed for 4 h at 5°C and 24 h at 22°C. Distinct lipid bodies and protein storage vacuoles could not be observed (*). Mean separation tests detected significant differences in cross sectional areas for *C. wrightii* (*bars labeled a, b*) and *C. lanceolata* (*bars labeled y, z*) at the $\alpha = 0.05$ level

Discussion

We have shown that germination success of seeds from two species of *Cuphea* can be manipulated predictably by temperature treatments that control triacylglycerol phase in dry seeds. Lipids within the seeds of the two species contain differing proportions of capric and lauric acids which confer a ~10°C difference in the melting temperature of the triacylglycerols. Seeds with crystallized triacylglycerols do not survive imbibition. These findings are important for genebank operators who have been unable to store germplasm from tropical plant species and for oilseed producers in temperate areas who use cultivars with high amounts of saturated or monounsaturated fatty acids.

Our research on triacylglycerol-mediated damage during cold exposure shares tenets with past research linking phase changes in polar lipids with chilling or

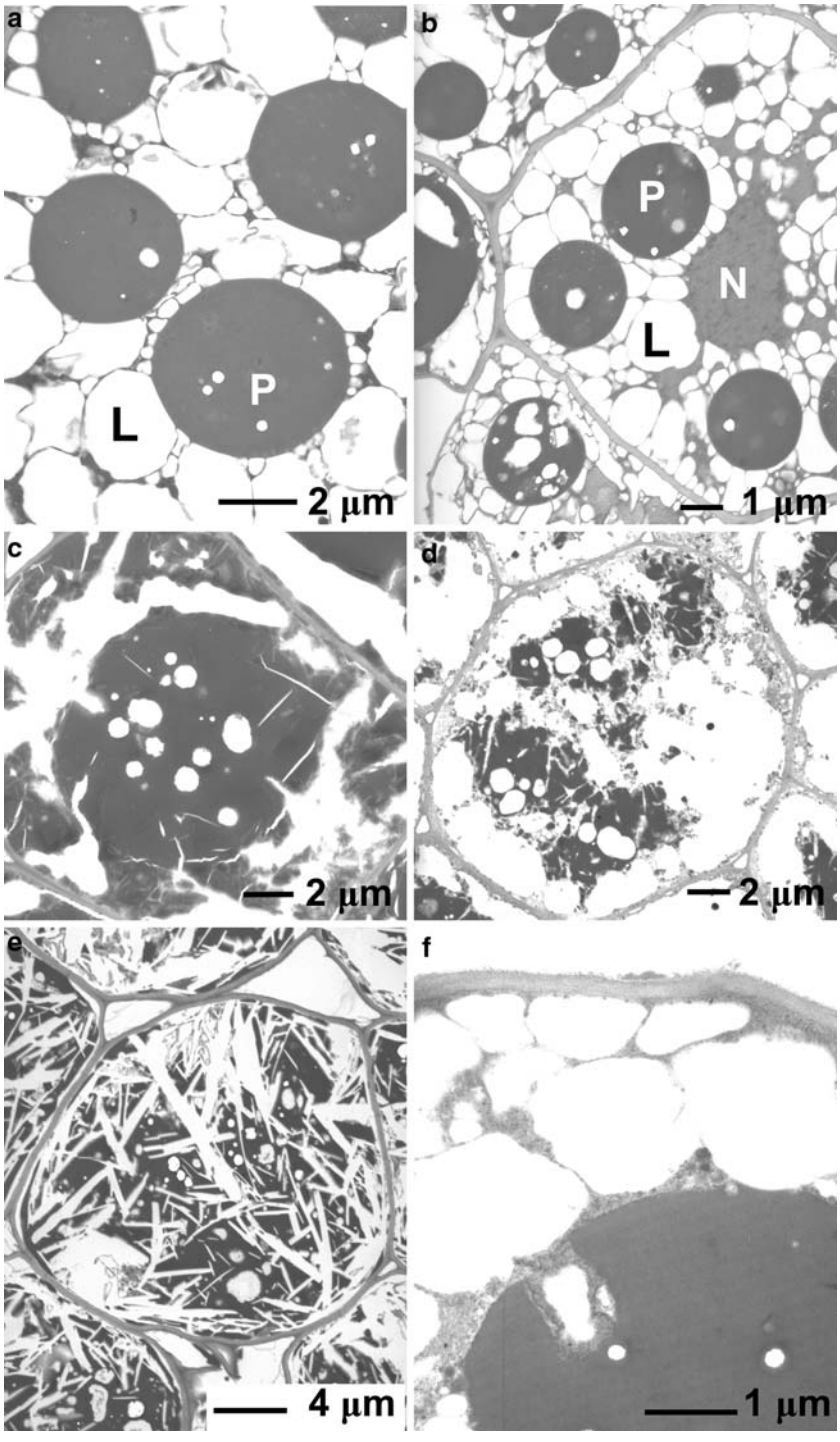

Fig. 6 Ultrastructure of cells of *Cuphea wrightii* (**a, c, e**) and *C. lanceolata* (**b, d, f**) seeds cooled to −80°C and imbibed at 5°C for 4 h (**a, b**), 24 h (**c, d**), or 5°C for 4 h and then 22°C for 24 h (**e, f**) prior to embedding. *P* protein storage vacuoles; *L* lipid body; *N* nucleus

desiccation damage (e.g., Lyons et al. 1979; Crowe et al. 1989; Crowe and Crowe 1992; Steponkus et al. 1995; Hoekstra et al. 2001). Unlike systems studied earlier, the plasma membrane does not appear to be the site of damage in imbibing *Cuphea* seeds. *Cuphea* seeds are tolerant of desiccation and so would likely possess sufficient protective mechanisms to avoid many of the hypothesized changes in membranes during exposure to low temperature or moisture. Further, the seeds did not exhibit the classic physiological symptom of membrane damage, leakage of cellular constituents including electrolytes, when they were exposed to lethal temperature treatments (Table 2), suggesting that the plasma membrane retained function even when intracellular constituents were disrupted. The similar transition temperatures for the polar lipid fraction of the two species, predicted from fatty acid compositions (Table 1), do not explain the difference in

temperature response between the two species. Presumably the polar lipids would be in the liquid crystalline phase at the imbibition temperatures studied here (Table 1), and so phase transitions within membranes are a less likely mechanism of damage in *Cuphea* seeds. Nonetheless, the idea that water interactions with crystallized lipid are damaging has precedence in the seminal work on hydration of gel phase polar lipids, published in the imbibitional chilling literature (Crowe et al. 1989; Hoekstra et al. 2001).

All intracellular structure was lost in *C. wrightii* seeds that were cooled to < -18°C, rewarmed to ≤ 22°C and imbibed. Triacylglycerols remained crystallized in seeds receiving this type of treatment. Cellular disintegration was evident during early imbibition (Figs. 2c, d, 4a, 6c), and suggests that damage occurred during the transition from an anhydrous to fully hydrated state. The same type of damage was rarely observed in cells of *C. lanceolata* seeds (Table 2, Fig. 6d), probably because their triacylglycerols required extreme cooling to −80°C to crystallize and readily melted when seeds were warmed to room temperature (Fig. 1). Visual evidence of cell damage in *C. lanceolata* seeds imbibed at 5°C was not observed initially (Fig. 6a, b), but took time to develop (Fig. 6c, d). Often the rapid cellular disintegration observed in imbibing *C. wrightii* seeds containing crystallized triacylglycerols was not observed in *C. lanceolata* seeds imbibed at 5°C, even though this treatment was lethal to the seeds (Table 2). In many cases, lethally treated cells of *C. lanceolata* seeds exhibited a disorganized ultrastructure, which progressed with imbibition time. The timing and temperature dependence of cell disruption in *Cuphea* seeds during early imbibition suggests that it arises from biophysical rather than metabolic mechanisms. The delayed appearance of massive cellular disruption in seeds imbibed at 5°C suggests that the primary lesion has not yet been detected.

The reversibility of conditions that impose damage provides important insight into the mechanism causing cell perturbations. Heating seeds could reverse potentially lethal effects of cooling dry seeds to temperatures that melted triacylglycerols prior to imbibition (heat pulses to 45°C and 22°C were required for *C. wrightii* and *C. lanceolata*, respectively). Even though germination was normal in seeds receiving this brief heat pulse, cell ultrastructure within *C. wrightii* seeds was different from that of the controls. After the heat pulse, protein storage vacuoles were smaller and lipid bodies were larger (Fig. 5) and electron dense material was mixed into the triacylglycerol matrix (Fig. 2e, f). These cellular rearrangements are consistent with the close association of protein and lipid bodies previously observed (Fernandez and Staehelin 1987) and perhaps suggest that protein reserves were subdivided and redistributed into the lipid bodies. One may speculate that the new oil bodies arise from a change in physical interactions among cellular components during crystallization and melting, and that these forces are different than those present during liposome and protein storage vacuole biogenesis that lead to controlled deposition of reserves during embryogenesis. Interestingly, heat pulses delivered after imbibition begins are ineffective at restoring viability. This supports the conclusion that the primary lesion for the type of damage we are reporting occurs during the initial stages of imbibition and is not apparent from the electron micrographs presented here.

We speculate that the lethal effect of water on crystallized triacylglycerols arises from an altered balance of hydrophilic and hydrophobic interactions that are necessary when anhydrous cells transition to an aqueous environment. The increased hydrophobicity of the crystallized triacylglycerols when water enters the cell may promote preferential interactions of liposome membrane components with other membrane systems and cause massive decompartmentalization. Steponkus and colleagues proposed interlamellar interactions among polar lipids as a mechanism of damage when unprotected cells were severely desiccated during exposure to subzero temperatures (Steponkus et al. 1995) and these interactions have also been proposed to explain lateral movement of enzymes among organelles (Fernandez and Staehelin 1987). Alternatively, oleosin orientation in lipid bodies may be affected when triacylglycerols crystallize, thereby affecting the stability of the organelle and the mode in which it hydrates during imbibition.

Conclusions

Seeds of some species are lethally damaged during imbibition following cold storage, and this sensitivity has hindered their preservation in genebanks. We have linked the temperature combinations that impart damage to the phase behavior of triacylglycerols within seeds of *Cuphea* species. Severe cellular perturbations occur early during imbibition of sensitive seeds and are irreversible once imbibition commences. Imbibition of seeds with crystallized lipids potentially alters intermolecular interactions that are fundamental to cell organization and stability in the hydrated state. Continued research in this area is needed to ensure optimum storage conditions for germplasm and to promote stand establishment of seeds with altered lipid compositions.

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